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CTNNA3 discordant regulation of nested *LRRTM3*, *implications for autism spectrum disorder and Tourette syndrome*

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ABSTRACT

Autism Spectrum Disorder (ASD) and Tourette syndrome (TS) are poorly understood neurodevelopment disorders with strong male bias, high heritability and complex genetic heterogeneity. *IMMP2L* and *CTNNA3* are associated with ASD and TS and both harbour nested genes *LRRN3* and *LRRTM3*, respectively, that demonstrate independent association with ASD. *LRRTM3* encodes a trans-synaptic ligand for neurexin 1 (NRXN1). NRXN1 has been recurrently disrupted in ASD and TS suggesting that complex overlapping pathogenetic processes are at play in these two disorders.
Methods: The *in vitro* expression levels of the overlapping gene sets *IMMP2L-LRRN3* and *CTNNA3-LRRTM3* were quantified using comparative PCR (C_TPCR) before and after targeting each of the harbouring genes with siRNA. An *Immp2l*-gene trap (*Immp2l*^{GT}) mouse strain was developed to test expression effects on the nested *lrrn3* gene.
Results: Reduced expression levels of *IMMP2L in vitro* and of *Immp2l* in *Immp2l*^{GT} mice had no observable effect on the expression level of their nested genes *LRRN3* and *lrrn3*, respectively. In contrast, the siRNA mediated down regulation of *CTNNA3* levels resulted in concurrent increases in the expression level of its nested gene *LRRTM3*.
Conclusion: The discordant regulation of *LRRTM3* by *CTNNA3* may help explain its association with ASD and TS. *LRRTM3* completes the set of neurexin trans-synaptic ligand gene families associated with ASD and TS reinforcing the status of the NTSC as a mutation hotspot for TS and a strong point of molecular overlap between TS and ASD.

1. Introduction

Autism Spectrum Disorder (ASD) is characterized by deficits in social interactions and communication as well as restrictive and/or repetitive behaviours and interests, while Tourette Syndrome (TS) is characterized by multiple motor and one or more vocal tics that have been present for more than a year (American Psychiatric Association, 2013). Overlap of ASD and Tourette Syndrome (TS) is reported in around 5 to 10% of cases while up to 20% of ASD cases have tics, indicating overlapping aetiologies and mixed molecular determinants of behaviour (Baird et al., 2006; Clarke et al., 2012).

Global genomic analyses indicate extreme genetic heterogeneity in ASD with hundreds of loci implicated (Abrahams and Geschwind, 2008b; Cristino et al., 2013; O'Roak et al., 2012). ASD genes encode 13 distinct protein modules that include cell-cell adhesion and synaptogenesis (Cristino et al., 2013). Interestingly, the *CTNNA3* gene and its nested gene *LRRTM3* both associate with ASD and *CTNNA3* with TS (Bacchelli et al., 2014; Sousa et al., 2010; Sundaram et al., 2010; Wang et al., 2009; Weiss et al., 2009) and both have association with cell-cell adhesion and synaptogenesis (Clarke et al., 2012) thus raising the specter of a regulatory relationship between these two overlapping genes. Furthermore, LRRTM3 is part of the neurexin trans-synaptic connexus (NTSC), a mutation hotspot for TS and a strong point of molecular overlap between TS and ASD. NRXNs 1-4 and the full complement of known neurexin trans-synaptic ligand families have now been associated with TS and/or ASD: Neuroligins (NLGNs); Cerebellin precursor (CBLN/GRID) complexes; and Leucine rich repeat transmembrane proteins (LRRTMs) (Clarke et al., 2012).

Similar questions also arise regarding a possible regulatory relationship between a second overlapping gene set *IMMP2L-LRRN3* and its association with ASD and TS (Clarke et al., 2012; Maestrini et al., 2010; Pauls and Leckman, 1986). LRRN3 is structurally related to LRRTM3 but there is no evidence that LRRN3 is in any way associated with the NTSC.

The recurrent disruption of *IMMP2L* and *CTNNA3* in ASD and TS (Bacchelli et al., 2014; Bertelsen et al., 2014; Casey et al., 2012; Chen et al., 2013; Patel et al., 2011; Petek et al., 2007; Petek et al., 2001; Sundaram et al., 2010) prompted this study to determine if reduced

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expression of *IMMP2L* and *CTNNA3* affects the expression of their nested genes *LRRN3* and *LRRTM3*, respectively.

2. Methods

2.1. Ethics statement

Studies involving human cell lines and mice were approved by the Institutional Human Ethics Committee and Animal Ethics Committee, respectively.

Oligonucleotide primers (Sigma-Aldrich) were designed and selected for their reliable PCR amplification of each of the genes of interest (Table 1). Using comparative PCR (C_TPCR) the H4 Neuroglioma cell line (American Type Culture Collection) was shown to express moderately high levels of *CTNNA3* and *LRRTM3* but not *LRRN3*. The PA Astrocyte cell line acquired from Nadi Braidy (University of NSW, Australia) expressed both *IMMP2L* and its nested gene *LRRN3*, albeit, *IMMP2L* was expressed at much lower levels compared to *LRRN3*.

Cell lines were cultured to 80% confluence in RPMI cell culture medium supplemented with 10% fetal calf serum (FCS). Cells were treated briefly with trypsin, washed in RPMI with FCS and then plated at a density of 50,000 cells per well in a 12 well plate format and cultured for 24 h prior to siRNA transfection in FCS free medium. Prior to transfection siRNAs (Table 2) were diluted to 5 nM stocks in nuclease free buffer. siRNA [10pM] were incubated with Lipofectamine RNAiMax transfection reagent in serum free medium according to manufacturer's instructions (Life Technologies). Assays were performed in triplicate with and without siRNA in serum free medium. After 6 h the FCS was restored to a concentration of 10% and cells incubated for a further 18 h prior to replacing the media and culturing for a further 24, 48 and 72 h before harvesting for analysis.

RNA was prepared using the PureLink RNA minikit (Life Technologies Australia Pty Ltd) according to manufacturer's instructions using 18 gauge needles for DNA fragmentation. RNA was quantitated using the NoniDot 2000 system. cDNA was synthesised using the SSIII 1st Strand QPCR Supermix according to manufacturer's instructions (Life Technologies Australia Pty Ltd). C_TPCR was performed using SYBR select Master Mix (Life Technologies) in a 384 well MicroAmp plate format using the ViiA™ 7 Real-Time PCR system for 40 cycles and 57 °C annealing. All siRNA assays were analysed in triplicate wells using RT Profiler PCR Array Data Analysis version3.5 software. Assay integrity was monitored by tracking the amplification of *GAPDH* and *β-ACTIN* in real time.

Table 1
Oligonucleotide primers.

LRRTM3-F	GAATACGCAGACTCAAAGAG
LRRTM3-R	TTCCGTAAATTTGTCACAGG
CTNNA3-F	AAATTGTCAAATTGCAGCC
CTNNA3-R	CTTGTAATGTCATCTACGGC
LRRN3-F	GGCAACATTTATTTAACATGCTCCACAGC
LRRN3-R	TCCCATGCTTCTTCAGTATTTGCAGGA
IMMP2L-F	GCCTTCTTTGAATCCTGGG
IMMP2L-R	CCTATGGTTCTGACAATATCTCC
GAPDH-F	TGACCCTTCATTGACCTCA
GAPDH-R	AGTCCTTCCACGATACCAAA
Immp2l-F	TTGCAAAGGCTTCTTCGTG
Immp2l-R	TCAAAGAAGGCTGCATTGAC
Lrrn3-F	TCAGCCAAGGAACTGAGCAAGC
Lrrn3-R	TCTCTTTCTTCCCAGGTCCCAGA
Gapdh-F	ACCACAGTCCATGCCATCAC
Gapdh-R	CACCACCCTGTTGCTGTAGCC
P1 (Immp2l-DNA-F)	CTCTTGGCTGTATTTGTTTACC
P2 (Immp2l-DNA-R)	CTAGTTGGAAGATTTGGAAAGG
P3 (Immp2lGT-DNA-R)	CCAATAAACCCTCTTGCAGTTGC
Sry-F	TTGTCTAGAGAGCATGGAGGGCCATGTCAA
Sry-R	CCACTCCTCTGTGACACTTTAGCCCTCCGA

Table 2
Custom siRNAs.

CTNNA3 (terminal exon siRNAs)	
CTNNA3–1	GGCACCUAACCAGGCAGAAAUGUAU AUACAUUUCUGCCUGGUUAGGUGCC
CTNNA3–2	GGUUUCCAAUGUGUUAGAGAGUUCU AGAACUCUCUAACACAUUGGAAACC
CTNNA3–3	UAACUGCAUGAACUGUACCAUUGAA UUCAAUGGUACAGUUCAUGCAGUUA
CTNNA3 (splice site siRNAs)	
CTNNA3–4	CAGACAGCUCCGCAAGGCUAUUAUA UAUAAUAGCCUUGCGGAGCUGUCUG
CTNNA3–5	CAGGCUUGUAGAGGUGGCAAAUCUU AAGAUUUGCCACCUCUACAAGCCUG
IMMP2L (terminal exon siRNAs)	
IMMP2L -1 (337)	GAAGUACACCGUGGUGACAUUGUAU AUACAAUGUCACCACGGUGUACUUC
IMMP2L -2 (474)	UGGUCACAUCUGGGUUGAAGGUGAU AUCACCUUCAACCCAGAUGUGACCA
IMMP2L -3 (1074)	GGUAGAACAAGAAGGAACACAUAUU AAUAUGUGUUCCUUCUUGUUCUACC
Control siRNAs	
Control siRNA1	CAUAAUGGGAUUCACCUCCAAAUUA UAAUUUGGAGGUGAAUCCCAUUAUG
Control siRNA2	CAUGUUCAACACUUUGUCAACUGAA UUCAGUUGACAAAGUGUUGAACAUG
Control siRNA3	GAGCUAAUUGGGAAGACUUACAUAA UUAUGUAAGUCUUCCCAAUUAGCUC
Scrambled siRNA	GGGAAGAGGUCACAUACCAUUCCCA UGGGAAUGGUAUGUGACCUCUUCCC

Changes in gene expression levels were determined relative to changes in expression levels after exposure to a scrambled siRNA (Table 2).

Results are presented as bar graphs that depict the mean relative gene expression levels with error bars representing the standard deviations obtained from the averages of 3 experiments where each experiment is assessed in triplicate. Relative expression values are obtained by the delta delta Ct method, where *GAPDH*/*Gapdh* is utilized as the control gene (delta Ct) where results are normalised to one control well (delta delta Ct). Relative expression values are plotted on the y-axis. The different genes and their expression level measurements on the x-axis have different shading as depicted in boxed insets.

2.2. Generation of the *Immp2l*^{GT} mouse strain

Gene Trap (GT) technology was selected for its potential to retain residual levels of target gene expression with the aim of avoiding the infertility of the full *Immp2l* −/− knockout mouse developed in 2008 with its associated difficulties in breeding and behavioural testing (Lu et al., 2008). The *Immp2l*^{GT} mouse strain was developed from ES cells (ES clone IST11147G7, Texas Institute of Genomic Medicine, Texas, USA) harbouring a single gene trap cassette within the first intron of the *Immp2l* gene (Fig. 1A) using the method described by Cotton et al. (2015) (Australian Phenomics Network Monash University). To confirm maintenance of the gene trap cassette in clonal cell culture and to monitor its segregation in *Immp2l*^{GT} mice a multiplex PCR was designed for DNA derived from ES cells and mouse tail sections using a REDExtract-N-Amp Tissue PCR kit (Sigma Aldrich Australia) and *Immp2l* and gene trap specific oligonucleotide primers P1, P2 and P3 (Table 1 and Fig. 1A). PCR was performed in a microwell plate format using the ViiA™ 7 Real-Time PCR system for 35 cycles with 58 °C annealing. Primer P1 (located ~30.2 kb downstream of *Immp2l* exon ENSMUSE00000778835) and P2 (located ~30.5 Kb downstream of *Immp2l* exon ENSMUSE00000778835) amplify a product of 331 bp from the wild type allele. Primer P1 (also located 5′ to the 5′ LTR region of the gene trap cassette) and P3 (located in the 5′ LTR of the cassette) amplify a product of 179 bp from the targeted allele (Figs 1A and B).